CONCISE COMMUNICATION

# Susceptibility of strains of *Streptococcus agalactiae* to macrolides and lincosamides, phenotype patterns and resistance genes

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The Group B streptococcus (*Streptococcus agalactiae*) is a pathogen of increasing importance in human disease. We therefore studied the susceptibility of clinical isolates of *S. agalactiae* to penicillin G, erythromycin, azithromycin and clindamycin using National Committee for Clinical Laboratory Standards methodology, and we also determined the phenotypes of macrolide-lincosamide susceptibility and the resistance genes implicated in a group of selected isolates of the different phenotypes. We used 221 isolates collected between 1997 and 1999 in two Health Authority Areas in Móstoles and Granada, Spain. The minimal concentration for 90% inhibition ($MIC_{90}$) for penicillin G was 0.12 mg/L and all the isolates tested were susceptible. One hundred and eighty-five (83.7%) were susceptible to erythromycin and azithromycin and 191 (86.4%) were susceptible to miocamycin and clindamycin. Twenty-three isolates (10.4%) had a constitutive $MLS_B$ phenotype, seven (3.2%) an inducible phenotype, and six (2.7%) an M phenotype. All except one of the $MLS_B$ phenotype isolates tested ($n = 23$) carried *erm* genes; in two strains with the *mef* (A) gene, all the M phenotype ($n = 6$) isolates tested carried *mef* genes, while *erm* and *mef* (A) genes were absent in all the macrolide-lincosamide-susceptible ($n = 12$) isolates tested. In our environment, resistance to macrolide and lincosamide in *S. agalactiae* was present in 10–16% of the isolates. The majority of resistant strains had the $MLS_B$ phenotype.

**Keywords** Susceptibility, *Streptococcus agalactiae*, resistance genus

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The Group B streptococcus (*Streptococcus agalactiae*) is a pathogen of growing importance in human pathology. Infections in adults produced by this pathogen are currently infrequent. There are few recent publications on this subject and the majority refer to patients with risk factors such as diabetes mellitus, neoplasia, or hepatic insufficiency [1] or extreme age [2]. It has been implicated in bacteremia, pneumonia, endocarditis, urinary tract infections, septic arthritis, skin infections, empyema, gangrene and puerperal infection, but it is of principal interest as the most important cause of neonatal sepsis [1,3].

$\beta$-Lactam antibiotics, and specifically penicillin G, have been, and are currently, the treatment of choice for these processes since their continued use has not modified the in vitro susceptibility of these micro-organisms, although in some strains tolerance has been reported [4]. However, suspected or confirmed allergy to penicillin has meant that, in a number of clinical cases, treatment with penicillins has to be ruled out, as well as, as a precautionary measure, all $\beta$-lactam agents [1,3].

Since their introduction in the 1950s, resistance to macrolides and lincosamides remained stable throughout the world until the middle of the 1990s, when it rose in the USA [4–10]. Two resistance mechanisms have been described: modification by methylation of the antibiotic target, the ribosome, and the active efflux of the antibiotic across the membrane. Modification of the target is produced by its alteration when a methylase coded

by the *erm* (erythromycin ribosome methylation) genes introduces two methyl residues in an adenine of 23S rRNA. This mechanism affects 14-, 15- and 16-membered macrolides and also lincosamides and streptogramin B antibiotics. A second and recently described gene in *Streptococcus pyogenes* is responsible for the active efflux across the membrane of 14- and 15-membered macrolides but not of 16-membered macrolides or lincosamides [11]. Subsequently this gene has been found in strains of *Streptococcus pneumoniae* and other streptococci and is known as the *mef*(A) (macrolide efflux) gene. It is thought that it codes a hydrophobic protein of the membrane which uses the energy of the proton pump and actively expels the antibiotic from the interior of the cell [11].

In view of the few studies performed in Europe concerning the antibiotic susceptibility of *S. agalactiae* to macrolides, the main aim of this study is to ascertain the susceptibility of *S. agalactiae* strains to different macrolides and lincosamides, to determine their phenotypic patterns and their resistant mechanisms.

Over the period 1997–99 we collected 221 isolates of *S. agalactiae*. The first group of bacteria was obtained from clinical samples received by the Microbiology Service of the Hospital of Móstoles ($n = 171$) and their origin was as follows: 37 from urine, two from vaginal exudates and seven from rectal exudates of pregnant women, five from cerebrospinal fluid, two from urethral exudates, one from ear exudate, one from conjunctival exudate, one from sputum and 41 from blood cultures (the latter isolated since 1990). The second group of bacteria ($n = 50$) was from vaginal exudates of pregnant women treated in the Hospital Virgen de las Nieves of Granada. Strains were identified by standard criteria: colonies of Gram-positive cocci, $\beta$-haemolytic on sheep blood agar, catalase negative, that react with Lancefield group B antiserum. All strains were kept frozen at $-40\,^{\circ}C$ in cryotubes which contained 10% skimmed milk.

The antibiotics tested were: penicillin G (Sigma Chemical Co., St Louis, MO, USA), a 14-membered macrolide, erythromycin (Sigma Chemical Co), a 15-membered macrolide, azithromycin (Pfizer Inc., New York, NY, USA), a 16-membered macrolide, miocamycin (diacetyl-midecamicin; Menarini, Barcelona, Spain), and a lincosamide, clindamycin (Sigma Chemical Co).

The antibiotics were incorporated into medium in a $\log_2$ dilution series from 0.008 to 2 mg/L for penicillin G and from 0.06 to 64 mg/L for macrolides and the lincosamide. The minimum inhibitory concentrations (MICs) of each antibiotic were determined by the agar dilution method. Mueller–Hinton agar medium with 5% sheep blood was used according to the National Committee for Clinical Laboratory Standards (NCCLS) criteria [12]. Inocula were prepared by diluting bacterial suspensions equivalent in turbidity to 0.5 McFarland standard, to provide approximately $10^4$ colony-forming units per spot when applied by a Steer's replicator (Craft Machine Inc., Chester, PA, USA).

The plates were incubated overnight at 35 °C in an atmosphere containing 5% carbon dioxide. The range of interpretative categories for each antibiotic were those recommended by NCCLS in the 2000 supplement [13]. The MIC breakpoints for miocamycin were $\leq 1$ mg/L for susceptible and $>4$ mg/L resistant, as defined by the Comité de l'Antibiogramme de la Societé Française de Microbiologie. *Staphylococcus aureus* ATCC 29213 and *S. pneumoniae* ATCC 49619 were used as quality control strains.

Discs containing erythromycin (15 μg) or clindamycin (2 μg) were used to identify antibiotic-resistance phenotypes. Different phenotypes of macrolide-lincosamide-streptogramin B ($MLS_B$) resistance were recognized in accordance with the description of Seppälä et al. [14]. After 24 h of incubation at 35 °C, blunting of the clindamycin inhibition zone proximal to the erythromycin disc was taken to indicate inducible resistance. Resistance to clindamycin (confirmed by the agar dilution method) with no blunting of the clindamycin inhibition zone indicated constitutive resistance. The recent resistance phenotype, designated M phenotype, was characterized by susceptibility to clindamycin with no blunting of the inhibition zone around the clindamycin disc.

Forty-one selected strains of the different phenotypes were used to determine the presence of *erm* and *mef* genes: 12 susceptible strains, six with the M phenotype, and 23 with the $MLS_B$ phenotype. The MLS resistance mechanism was determined by polymerase chain reaction (PCR) by amplification of *erm* genes, using degenerate *erm* primers ($E_1$ 5′-GARATIGGIIIIGGIAAGAGGICA-3′; $E_2$ 5′-AAYTGRTTITTIGTRAA-3′). The efflux-pump mechanism was determined by PCR using primers and specific conditions for amplification of *mefA/E* genes ($A/E_1$ 5′-AGTATCATTAATCACTAGTGC-3′; $A/E_2$ 5′-TTCTTCTGGTACTAAAAGTGG-3′)

**Table 1** In vitro susceptibility of 221 recent *Streptococcus agalactiae* isolates to penicillin G, clindamycin and three macrolides

| Antibiotic | MIC (mg/L) | | | Susceptibility rates (%) |
|---|---|---|---|---|
| | Range | $MIC_{50}$ | $MIC_{90}$ | |
| Penicillin G | ≤0.03–0.12 | 0.06 | 0.12 | 100 |
| Erythromycin | ≤0.06- >64 | ≤0.06 | 8 | 83.7 |
| Azithromycin | ≤0.06- >64 | ≤0.06 | 8 | 83.7 |
| Miocamycin | ≤0.03- >64 | 0.5 | 2 | 86.4 |
| Clindamycin | ≤0.03- >2 | ≤0.06 | 1 | 86.4 |

MIC breakpoints for susceptibility (mg/L) ≤0.12 for penicillin G, ≤0.25 for erythromycin and clindamycin, ≤0.5 for azithromycin, and ≤1 for miocamycin.

[11]. Positive and negative controls from our collection were used in all cases. Genomic DNA for PCR reactions was obtained by the Instagene matrix system (BioRad, Hercules, CA, USA) according to the manufacturer's instructions.

Table 1 shows the range of MIC, $MIC_{50}$, $MIC_{90}$, and the percent susceptible to the five antimicrobial agents tested.

Penicillin G was the most active antibiotic with an $MIC_{90}$ of 0.12 mg/L. Resistance rates for macrolides were 16.3% for erythromycin and azithromycin, as representatives of 14- and 15-membered macrolides, respectively, 13.6% for miocamycin, as a representative of a 16-membered macrolide, and 13.6% for clindamycin, the lincosamide tested.

Of the strains studied, 185 (83.7%) showed a phenotype susceptible to macrolides-lincosamides, 30 (13.6%) had an $MLS_B$ resistance phenotype [23 (10.4%) a constitutive phenotype and seven (3.2%) an inducible phenotype] and six (2.7%) had an M resistance phenotype.

Forty-one strains were selected for PCR study of the resistance genes. None of the resistance genes searched for was found in either of the 12 susceptible strains. In 22 out of 23 strains with the $MLS_B$ phenotype an *erm* gene was found; in two strains it was found with the *mef* (A) gene. In the six strains with the M phenotype an *mef* (A) gene was found.

All strains with the $MLS_B$ constitutive phenotype ($n = 23$) had MIC of >64 mg/L for all the macrolides and the lincosamide tested, and the seven strains with the $MLS_B$ inducible phenotype were resistant to erythromycin with MICs of 4 mg/L, susceptible to clindamycin (MICs of 0.12 mg/L) and had an intermediate resistance to miocamycin (MICs of 2 mg/L).

Our findings demonstrate that *S. agalactiae* isolated from a variety of patients with invasive or non-invasive infections or from healthy pregnant woman remain uniformly susceptible to penicillin G. Although increased use of intrapartum chemoprophylaxis, theoretically, may promote the development of group B streptococcal resistance to penicillin, we and other investigators have failed to identify this phenomenon.

In our review of the literature for penicillin, erythromycin and clindamycin, the median MICs remained stable from 1957 to 1995 and these results have been confirmed by several investigators with only minor disparities between the MICs, probably due to methodological differences.

Since 1995, resistance to macrolides and lincosamides has risen, at least in the USA, where resistance values to erythromycin vary from 7 to 21% and to clindamycin from 3 to 15% [2,7,9,10]. However, in Europe, there are few data concerning an important increase in resistance to macrolides and lincosamide [15,16]. Two very recent papers [15,16] displayed results similar to ours. Ruess et al. [15] showed increasing macrolide resistance in recent years. In our study, with strains very recently obtained in two Spanish centers, we found a high percentage of resistance to macrolides and lincosamides, similar to that reported in the USA. The identification of resistant strains suggests that these agents should be used with caution in the prophylaxis or treatment of *S. agalactiae* infection in patients allergic to β-lactams.

Traditionally the macrolides, and specifically erythromycin, have been considered the treatment and prophylaxis of choice against *S. agalactiae* in patients allergic to β-lactams [3]. The fact that resistance is increasing has led some authors to reconsider these criteria and to look for alternatives [1], recommending clindamycin instead of erythromycin, not only as a result of the increase of strains with M phenotype, susceptibility to clindamycin and resistance to erythromycin, but also because clindamycin crosses the transplacental barrier better, reaching higher levels in the fetus [1,10].

In our environment more than 13% of *S. agalactiae* strains are resistant to macrolides. The majority of these strains present an $MLS_B$ phenotype, mainly constitutive, due to *erm* genes and, in contrast with the strains of *S. pyogenes* [17], only 2.7% of *S. agalactiae* strains studied present an M phenotype of resistance due to the *mef* (A) gene, which produces efflux of some antibiotics. These data oblige us to consider the susceptibility to macrolides and lincosamides in each individual strain before using any of these antibiotics.

Periodic surveillance of the susceptibility of *S. agalactiae* to macrolides and lincosamides is therefore necessary to promote the appropriate therapeutic choices for infections caused by this species, especially in the groups of patients in whom this species is important.

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